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Research Article

IL-13 Has a Positive Inotropic Effect Associated with an Increase of Membrane Excitability on Healthy Rat Heart but not in Septic Rat Heart

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ABSTRACT

Introduction: Interleukin 13 (IL-13) is a cytokine produced during sepsis. The pro- and/or anti-inflammatory effects of IL-13 still remain not clearly stated, especially at the heart level. In this study, we evidenced the impact of IL-13 on (i) the heart contraction; and on (ii) the voltage-dependent Na^+ channels, $Na_V1.4$ and $Na_V1.5$, which are responsible for the membrane excitability, are essential for the excitation/contraction coupling.

Methods: Rat hearts were perfused *ex vivo* with IL-13 at 10ng/ml. The contractile force, heart frequency and coronary flow were recorded. The expression and translocation of $Na_V1.4$ and $Na_V1.5$ were analyzed by western blot after extraction of membrane and cytosol proteins from ventricular cardiomyocytes.

Results: Results showed that IL-13 induced an increase of the contractile force (+28.3%), as well as of both maximal speeds of contraction (+35.5%) and relaxation (+38.9%). We also demonstrated that IL-13 was acting via a pathway involving β1-adrenergic - adenylyl cyclase - PKA activation. An increase in sodium current was also shown to be regulated by the same pathway. The hearts perfused with IL-13 showed increased number of $Na_V1.4$ (+37.4%) and $Na_V1.5$ (+52.2%) at the membrane level, and the ratios of membrane/cytosol channels proteins were also increased after IL-13 perfusion for $Na_V1.4$ (+281.4%) and $Na_V1.5$ (+214.4%).

Conclusion: This study shows that IL-13 has a positive inotropic effect on perfused heart and that IL-13 can also increase $Na_V1.4$ and $Na_V1.5$ membrane targeting, therefore increasing the membrane excitability of the cardiomyocytes. However, IL-13 was shown to lose its inotropic effects in chronic septic hearts.

Introduction

Critical illness polyneuromyopathy (CIP) is one of the most frequently acquired pathology in patients admitted in intensive care units (ICU) [1]. The main and most dangerous symptoms are a decrease of muscle excitability and force. In many patients, the diaphragm can also be affected thus inducing difficulties in weaning from mechanical ventilation, and leading to a prolonged time in ICU, an increase in hospital costs and long-term sequels. In severe forms, CIP can lead to cardiac dysfunction [2]. In fact, during sepsis, acute heart dysfunction is one of the major predictors of morbidity and mortality [3]. It is characterized by an impairment of both left ventricular systolic and diastolic functions, associated with vasodilatation and decreased arterial and venous resistance thus inducing a decrease in arterial pressure [4, 5]. All these effects can lead to organ failure and eventually to heart failure. Even if a recovery occurs, sequels can be life lasting effects, thus impairing the quality of life of the patients. Many risk factors have been reported, especially sepsis and systemic inflammatory response syndrome (SIRS).

When sepsis occurs, mediators of inflammation, such as tumor necrosis factor alpha (TNF-α), interleukin-1beta (IL-1β) or 6 (IL-6) are released

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in the early stages of the illness and have pro-inflammatory effects. TNF-α has been reported to impair the membrane excitability in skeletal muscles by a rapid post-translational effect involving a protein kinase C (PKC) pathway activation and to cause a decrease of the cardiac contractility [6, 7]. Membrane excitability is the first step of the excitation-contraction coupling which regulates the muscle contraction in both skeletal and cardiac muscle. The impairment of the membrane excitability, in heart for example, lead to the decrease of the contraction force, reinforcing the blood pressure decrease and possibly leading to organ failure [8]. This is for this reason that we focused the present study on one aspect of this important mechanism, which is not much investigated in the muscle weakness occurred during CIP.

IL-13, a potent anti-inflammatory cytokine produced by Lymphocyte T helper 2 (Th2) cells, natural killer cells or mast cells, has been detected in septic patients [9, 10]. The severity of the symptoms was correlated to the levels of IL-13 [11]. Although IL-13 is involved in many immune and anti-inflammatory pathways in response to various illnesses and disorders, such as asthma, CLP, or endotoxemia, IL-13 can either have a pro- or anti-inflammatory function, depending on its location in the body and the illness type [11-15]. In example, IL-13 can act as a pro-inflammatory cytokine, especially in patients with asthma, as it can stimulate an eosinophilic inflammation and induce the contraction of the tracheal rings in the airway smooth muscle cells [13-15]. In the opposite, an animal model of sepsis induced by cecal ligation and puncture (CLP) showed that the production of endogenous IL-13 has a positive effect on the survival of the animals [12]. Moreover, in the diaphragm, IL-13 can also protect muscle contraction by preventing the decrease of force-frequency curves and twitch during endotoxemia [16]. However, its effects on healthy and septic hearts are still poorly understood. In fact, to our knowledge, there is no report of an intrinsic effect of IL-13 on rat heart. In the present study, our main focus was to characterize the effects of IL-13 on the function of a healthy heart by performing experiments on isolated perfused rat hearts and isolated cardiomyocytes. Those analyses focused on

i. Whether IL-13 could induce a positive inotropic effect associated with an increased excitability of the heart membrane; and
ii. determining which molecular pathway was activated by IL-13. Moreover, we tried to determine if IL-13 had a similar effect on septic heart in order to assess if IL-13 can induce a protective function of the heart function during sepsis.

Materials and Methods

I Animals

Female Wistar rats averaging 250g were used for the experimentation. All the animals were purchased from Janvier (Le Genest SaintIsle, France) and housed in the same location at 23°C, with a 12-hr daylight cycle and fed *ad libitum* with standard rat pellets. The experiments were carried out according to the recommendations of the European Community directive No. 2010/63/EU, which are equivalent to NIH recommendations. Experimental protocol, animal care, anesthesia, analgesia and sacrifice methods were approved by our ethical regional committee (CEFEA: "*Comité d'éthique Finistérien en expérimentation animale*" - departmental agreement No. A29-019-03) and validated by the French ministry in charge of animal experimentation and welfare (protocol No. 02076.01).

II Sepsis induction

The sepsis was induced by CLP according to method previously described [17, 18]. Briefly, rats were anesthetized with intraperitoneal (IP) injection of Xylazine (12mg/kg) and Ketamine (80mg/kg). After laparotomy, the caecum was carefully exposed then 10 punctures were performed in the caecum with a 21G needle, avoiding vessels. Gentle pressure was applied to the caecum in order to exude a small amount of feces. The cecum was sub-occlusively ligatured at its base below the colo-cecal valve and replaced into the abdominal cavity. The muscular layer and the skin were sutured separately with absorbable suture. Analgesia was carried out after the surgery by daily buprenorphine injection (0.1mg/kg) during the 7 days of procedure.

III Isolated Rat Heart Preparations and Recordings

The rats were deeply anesthetized with pentobarbital (60mg/kg), then killed by cervical dislocation and exsanguination. Rapidly, the heart and lungs were removed after sternotomy. Lungs were separated from the heart, and the aorta was cannulated according to the Langendorff technique. Heart was perfused at 37°C with modified Tyrode (NaCl 140 mM; KCl 5.4mM; $CaCl_2$ 1.8 mM; $MgCl_2$ 1.8 mM; HEPES 10 mM; glucose 11 mM all purchased from Sigma-Aldrich), adjusted at pH=7.4 with NaOH and oxygenated with 100% O_2. The Tyrode was supplemented with Heparin (1000 UI) during the first minutes of the perfusion in order to rinse the coronary system and to avoid thrombus formation. The heart was hooked by the apex to a strain gauge (100g WPI, UK) connected to a computer via a Biopac MP35 interface (CEROM, France). Hearts were allowed to beat spontaneously throughout the experiment. Biopac Student lab software (BSL Pro, CEROM, France) was used to record and analyze heart contractions for 30 sec periods, every 5 min. All the hearts were perfused at a constant pressure of 80 cm of water. Coronary flow was not imposed but was monitored by a ball flowmeter. After 25 min of control recording, recombinant IL-13 (Sigma-Aldrich) was added to the Tyrode to obtain a final concentration of 10ng/ml. The dose of 10ng/ml was chosen according to the publication of Colligan indicating that during sepsis the concentration of IL-13 was between 5-20ng/ml in human plasma [11]. For the inhibitors, we used 0.3µg/ml of KT5720, a selective PKA inhibitor (Alomone lab) added to the Tyrode, during 20 min before IL-13 addition. Similarly we used 100µM of SQ22536, a selective adenylyl cyclase (Ac) inhibitor (Abcam), and Esmolol at 1µM, a selective β1 adrenergic receptor inhibitor (Abcam). All hearts were perfused only once time with IL-13 or an inhibitor and IL-13 and were frozen for further analysis. Heart rate (bpm) and contraction force (g) were recorded and analysed with Biopac Student Lab software as well as the speeds of contraction ($+dF/dt_{max}$) and relaxation ($-dF/dt_{min}$) which were calculated in g/s, based upon the first derivative of contraction recordings. Coronary flow (ml/min) was recorded by using a fall flowmeter. At the end of experimentation, all hearts were frozen in liquid nitrogen until further analyses.

IV Western Blot analyses

Approximatively 200mg of the ventricles (around apex) were dissected then homogenized in Mem-PER™ Plus Kit (Thermo Fisher Scientific), according to the protocol given by the purveyor in order to separate cytosolic and membrane proteins. The protein content was measured with the Bradford method. Briefly, 5 µL of sample diluted (1:10) were

added to 250µL of Bradford reagent (Sigma-Aldrich) in 96 wells plate, and read at 595nm. Proteins were diluted in Laemmli Buffer 4x (Biorad) with 10% 2-Mercaptoethanol (Sigma-Aldrich). 40-120µg of proteins of each sample were run on 6% SDS-polyacrylamide gels then proteins were transferred onto nitrocellulose membrane (0.45µm, Millipore). The nitrocellulose membrane was then incubated in a blocking buffer (20mM Tris, pH 7.5, 150mM NaCl; TBS) supplemented with 0.05% v/v Tween 20 (TTBS) containing 5% w/v skimmed milk powder overnight à 4°C followed by 2x20 min washes in TTBS. Blots were then incubated with both anti-$Na_V1.4$ (1:200) (Alomone lab) and anti-$Na_V1.5$ (1:1000) (Sigma-Aldrich) in TTBS containing 1% w/v skimmed milk powder during 60 min at room temperature. The blots were washed 2x20min in TTBS and incubated with goat anti-rabbit IgG conjugated to horseradish peroxidase (HRP) (1:2000 dilution) (Abcam) for 60 min. Finally, blots were washed 2x20 min in TTBS, rinsed in TBS and exposed to ECL-reagents composed of 10ml buffer TrisHCL 100mM (Sigma-Aldrich) at pH=8.5, 3µl H_2O_2 50% (Sigma-Aldrich) 50µL of solubilized luminol (Sigma-Aldrich) in DMSO (Sigma-Aldrich) (44.3mg/ml DMSO) and 25µL of p-coumaric acid (Sigma-Aldrich) in DMSO (14.8mg/ml DMSO). Membranes were exposed for 1 min then analyzed by using a Vilber-Lourmat Fusion SL image acquisition system, and Image Studio Lite (LI-COR Biosciences) which allows precise quantification of the signal.

V Cardiomyocytes isolation

Langendorff system was used for the cardiomyocytes dissociation. A heart was obtained and perfused at 37°C with the modified Tyrode as described here above. Then, after 5min of contraction in normal Tyrode, the heart was perfused with Tyrode 0Ca (NaCl 140 mM; KCl 5.4mM; $MgCl_2$ 1.8mM; HEPES 5mM, glucose 10mM, taurine 20mM (Sigma-Aldrich); BSA at 1g/l (Euromedex)) adjusted at pH=7.4 with NaOH and oxygenated with 100% O_2 during five minutes, until contractions stopped. Then the heart was perfused with enzymatic oxygenated solution (70 ml of Tyrode 0Ca with 169mg of collagenase (Gibco) and 2mg of protease (Sigma-Aldrich). After 3 minutes, 1ml of 200mM $CaCl_2$ was added to the enzymatic solution and the perfusion carried out for an additional period of 15min. The heart was then removed from the system and placed in a Petri dish containing 1mL of the enzymatic solution. Atriums were removed and the ventricle minced. The medium containing cardiomyocytes was pipetted and added to 20ml of Kraftbrühe medium (KCl 70mM; glutamate 5mM; KH_2PO_4 20mM; $MgSO_4$ 5mM; $CaCl_2$ 0.08mM; EGTA 0.5mM; Creatine 5mM; Na_2ATP 5mM; taurine 20mM; HEPES 10mM all purchased from Sigma-Aldrich, BSA at 4g/l) then it was centrifuged at 1000 g during 1min. The pellet was resuspended with 20ml of Tyrode 0Ca then 200µl of $CaCl_2$ at 100mM was progressively added (aliquots of 40µl at 5 minutes intervals) to avoid calcium paradox.

VI Macro-patch clamp

As previously described (Guillouet *et al*., 2011), fast sodium currents were recorded by a macro-patch clamp technique at room temperature (22±1°C) in a cell-attached configuration with a GeneClamp 500B amplifier and a CV-5-100MU headstage (Axon Instruments, Foster City, CA). Micropipettes were pulled and polished from GC150TF-10 borosilicate glass (Harvard Apparatus, USA) with a DMZ-Universal puller (Zeitz Instruments, Germany). Pipettes had resistance averaging 1.7MΩ when filled with the recording solution (standard Tyrode). Voltage-clamp protocols and data acquisition were performed with WinWCP V4.17 (Whole Cell program, University of Strathclyde, Glasgow, UK) through a 16 bits data acquisition card (National Instruments, USA). Currents were low pass filtered at 5 kHz and digitalized at 100kHz. To eliminate residual capacitance, transient and leak currents, a P/4 procedure was used. Approach of the micropipette and realization of the seal was made by using hydraulic micro-manipulators (Narishige) on an inverted microscope (Olympus IX70) equipped with a progressive-scan digital camera (XC8500CE, Sony). Before recording, ventricle cardiac myocytes were placed in CsCl-containing medium (145 mM CsCl, 5mM EGTA, 1mM $MgCl_2$, and 10 mM HEPES, pH 7.4), which brings membrane to a resting potential value close to zero by blocking the potassium channels. Only the sodium current was recorded in the CsCl-EGTA containing medium. The holding potential was fixed at -100 mV. At this potential, almost all the sodium channels are in closed but activable state, leading few sodium channels to be nonconductive during the depolarizing pulses [19].

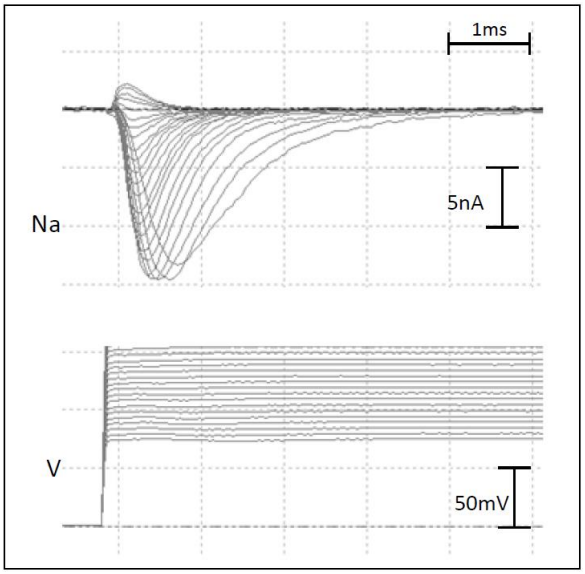


Figure 1: Example of sodium current recording, obtained during patch clamp experiment. It should be noticed that the WCP software give traces with inversed Y axis compared to usual representations. Sodium current recordings (upper trace) and imposed voltages (lower trace).

A pulse protocol was then applied to the patch membrane to record sodium currents (Figure 1). The stimulation protocol was a cycle of 20-ms test pulses ranging from -60 to +100mV in 10mV increments. Each test pulse was preceded by four hyperpolarizing pulses allowing leak current calculation (P/4 protocol). The interval between each test pulse was long enough (3s) to allow complete recovery of the sodium channels from slow inactivation. This protocol was repeated at least three times for each patch to ensure sodium current stability. A second program was used to determine the inactivation curve of the sodium channels by applying 50ms depolarizing pulses to the membrane before application of a 100mV test pulse from the holding potential, thus giving a membrane potential of 0mV. This value of the membrane potential was selected because it was close to the value corresponding to the maximum sodium current. The patches with no reliable peak current amplitude were discarded.

The currents were recorded in control condition on cardiomyocytes, then IL-13 (Sigma) was added to the medium at a final concentration of 10

ng/ml. Each cardiomyocyte served as its own control. This concentration was already used in previous studies, carried out in *peroneus longus*. The recordings were performed after 15 min following IL-13 addition. When used, the PKA inhibitor KT5720 was added to a final concentration of 0.3µg/ml, currents were recorded during 15min then IL-13 was added too and the recording was done 15min after the cytokine addition.

VII Data and statistical analyses

Maximal sodium current: $I_{Na\ max}$ represents the maximal sodium current (nA) related to membrane area under the patch pipette. The current-voltage relationship or *I-V* curve represents the relationship between imposed membrane potential and maximal sodium current registered. The maximal sodium conductance ($g_{Na\ max}$) is given by the slope of the quasilinear part of the *I-V* curve. Activation curve determination: the normalized activation curve was plotted according to the relationship: $g_{Na} / g_{Na\ max} = I_{Na}/[\ g_{Na\ max}(E_m-E_{Na})]$ where E_m is imposed membrane potential and E_{Na} is the Nernst potential for sodium which was determined from the *I-V* curve. The obtained activation curve can be fitted onto the Boltzmann equation: $g_{Na} / g_{Na\ max}=1/\{[\ 1+\exp(E_m-V_{m1/2})\ K_m\ ]\}$ thus allowing to determine $V_{m1/2}$ corresponding to the potential at which half of the channels are activated and K_m which is the slope factor. Fast inactivation curve determination: the fast inactivation curve was obtained by plotting the ratio $I_{Na} / I_{Na\ max}$ as a function of the membrane potential. I_{Na} was obtained according to the inactivation program previously described. The obtained inactivation curve was fitted onto a Boltzmann equation to determine $V_{h1/2}$ and K_h. Time constants determination: time constants corresponding to activation (τ_m) and inactivation (τ_h) of the sodium current were calculated by fitting $I_{Na\ max}$ onto the following Hodgkin-Huxley relation: $I_{Na\ max(t)}=A[1-\exp(-t/(\tau_m)\ ]^p.\ [h_{inf}-(h_{inf}-1).\exp(-t/\tau_h)\ ]$. Where A is the voltage-dependent part of the current, P is exponent of m, h_{inf} is the equilibrium value reached by *h* according to the potential, and *t* is the time. This was carried out by using the curve fitting menu of the WCP program.

All experimental data were analyzed by using Excel® (Microsoft, Redmond, WA) including Boltzmann curve fittings. All values are expressed by mean value ± SEM (Standard Error Mean). Statistical differences were determined by using paired Student's *t*-test or the Aspin-Welsh test after checking the distribution normality (Lilliefors test). A *p* value <0.05 was considered as statistically significant.

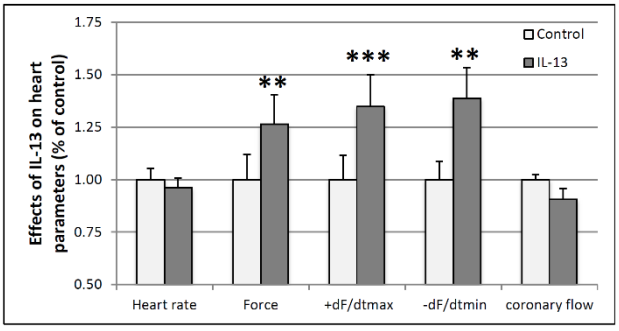


Figure 2: Cardiac parameters before and after IL-13 treatment at 10ng/ml for 15min in steady state hearts (n=7). Heart rate, contractile force, maximum speed of contraction (+dF/dt max) and relaxation (-dF/dt min), and coronary flow are represented. Each heart was its own control. Data are expressed by mean values ±SEM. Significant differences with **P<0.01 *vs* control, ***P<0.001 *vs* control.

Results

I Positive inotropic effect of IL-13 on the cardiac function of healthy rats occurs via the activation of β1-A - Ac - PKA pathway

To be able to accurately assess the effect of IL-13 on septic hearts, its effect on healthy hearts and the cardiac parameters had first to be determined. The impact of IL-13 on the heart rate, maximum contraction and relaxation speeds and coronary flow was assessed *ex vivo* by perfusing IL-13 in healthy isolated rat hearts by using the Langendorff system. All results for the hearts contraction are expressed as percentage of time 0 when we add the IL-13 at 10ng/ml in the Tyrode.

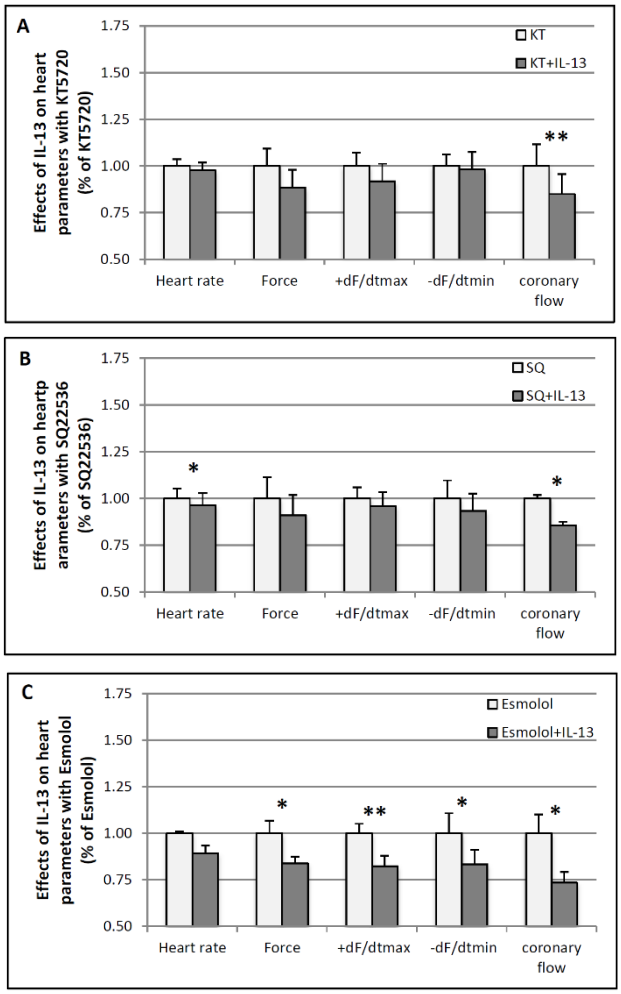
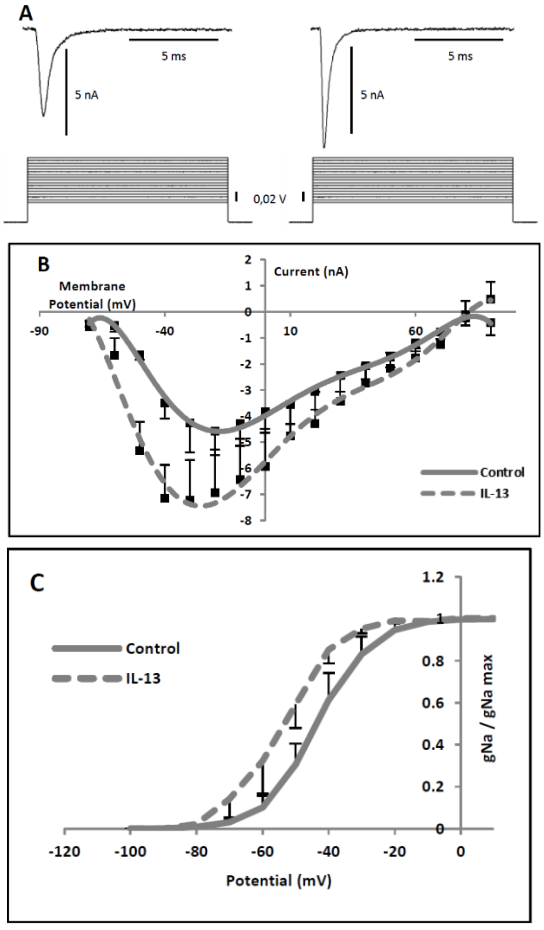


Figure 3: Effects of PKA inhibitor KT5720. (A) (n=4), Adenylyl cyclase inhibitor SQ22536 (B) (n=4), β1 adrenoreceptor adrenergic antagonist Esmolol (C) (n=3) on IL-13 perfused heart. Contractile force, maximum speed of contraction (+dF/dt max) and relaxation (-dF/dt min), and coronary flow are represented. Each heart represents its own control. Data are expressed by mean values ±SEM. Significant differences with *P<0.05 vs control, **P<0.01 vs control.

When the hearts were perfused with IL-13 for 15 min, the contractile force, the maximum contraction speed, and the maximum relaxation speed were increased by 26.5%, 34.7%, and 38.8% respectively (Figure 2). Yet, IL-13 had no effect either on the heart rate or the coronary flow. To determine which pathway could be involved in the positive inotropic

effect of IL-13 on normal hearts, selective inhibitors targeting either protein kinase A (PKA), adenylyl cyclase (Ac) or β1 adrenergic (β1-A) receptor were independently perfused in healthy hearts for 20 min prior to addition of IL-13 into the perfusion medium: KT5720, a PKA inhibitor, abolished all the effects of IL-13 on the contractile force, maximum contraction and relaxation speeds which were previously observed (Figure 3A). A slight (-15%) decrease of the coronary flow was also observed with KT5720 (Figure 3 A). SQ22536, an adenylyl cyclase (Ac) inhibitor had similar inhibitory properties than KT5720, concerning IL-13 inotropic effects, but in addition caused a decrease of the heart rate of 4% (Figure 3 B). This suggests that the inotropic effect of IL-13 is mediated via the activation of an Ac-PKA pathway. Finally, hearts were perfused with esmolol, a specific β1-A receptor inhibitor prior to adding IL-13. In this condition, the cytokine had a negative inotropic effect on heart suggesting that β1 receptors activation is involved in the positive inotropic action of IL-13 (Figure 3 C). In conclusion, Ac activation by IL-13 seems to be due to β1-A - Ac - PKA pathway activation.



E	Control	IL-13
$I_{Na\ max}$ (nA)	-4.80 ±0.87	-7.49 ±1.56*
$V_{½\ activation}$ (mV)	-46.43 ±4	-50.21 ±2.67 *
$V_{½\ activation}$ (mV)	-57.85 ±2.9	-60.46 ±4.33

F	Control	KT5720	KT5720 + IL-13
$I_{Na\ max}$ (nA)	-2.56 ±0.65	-2.53 ±0.66	-2.46 ±0.6

Figure 4: IL-13 at 10ng/ml increases sodium current in isolated cardiomyocytes. (A), upper trace: example of sodium currents recorded in control condition (left) and after IL-13 addition (right), lower traces: imposed membrane potentials. (B), effects of IL-13 on the sodium current voltage relationship (I/V curves) (n=5). (C), activation relationship of sodium current corresponding to the ratio of g_{Na}/g_{Namax} (n=5). (D), inactivation relationship of sodium current corresponding to ratio I_{Na}/I_{Namax} (n=5). (E), IL-13 increases $I_{Na\ max}$ and decreases the potential of $V_{1/2}$ for the activation curve (n=5). F, effects of IL-13 on I_{Namax} on cardiomyocytes incubated with KT5720. (n=7). Data are expressed by mean values ±SEM. Significant differences with *P<0.05 *vs* control.

II IL-13 induces an Ac-dependent increase in the cardiomyocytes membrane excitability and sodium current in healthy hearts.

To determine the effect of IL-13 on the excitability of the cardiomyocytes membrane, macropatch clamp measurements were performed on isolated ventricular myocytes from healthy rat hearts. This showed that IL-13, at a 10ng/ml concentration, induced an increase in the sodium current as shown on I/V curve (Figure 4 A and B). At this concentration, IL-13 induced an increase of the maximal sodium current -4.8±0.9 to -7.49±1.6 nA representing an increase of approximately 35% (Figure 4B). Interestingly, IL-13 caused a shift toward negative potentials of the activation curve which led to higher activation of the sodium channels for the same depolarizing potential (Figure 3 C). This was associated with a more negative potential for the $V_{1/2}$ activation, from -46.4±4 to -50.2±2.7 mV (Figure 4 E). In the opposite of what is observed for the activation curve, IL-13 had no effect on the inactivation curve of the heart membrane (Figure 4 D and E). To assess whether this increase in sodium current and membrane excitability were Ac-dependent, cardiomyocytes were pre-treated with PKA inhibitor KT5720 for 15min at 0.3µg/ml, leading to the blockade of the increase in sodium current induced by IL-13, suggesting that IL-13 effect on the membrane is Ac-dependent (Figure 4F).

III IL-13 induces the translocation of $Na_V1.5$ and $Na_V1.4$ from the cardiomyocyte cytosol to its membrane

Voltage-dependent Na^+ channels are membrane proteins responsible for the membrane excitability and essential in the excitation/contraction coupling process. The two main isoforms in ventricular myocytes are $Na_V1.5$ and $Na_V1.4$, representing respectively 77.5% and 8.7% of the population of the Na_V present in the ventricle [20]. To determine whether $Na_V1.5$ and $Na_V1.4$ could be involved in the positive inotropic effect of IL-13 on healthy hearts, western blots were performed on the membrane proteins, corresponding to the "active pool of Na_V", of the ventricular myocytes. We used hearts perfused with Tyrode without cytokine or inhibitor as a control group, and hearts perfused with IL-13 presented previously.

Membrane proteins were extracted then western blot targeting $Na_V1.5$ and $Na_V1.4$ performed. Hearts perfused with IL-13 showed an increase in $Na_V1.4$ and $Na_V1.5$ protein abundance at the membrane level of approximately +37% and +52% for $Na_V1.4$ and $Na_V1.5$ respectively (Figure 5A). To quantitatively assess the enrichment of membrane with $Na_V1.4$ and $Na_V1.5$ channels after perfusion of IL-13, membrane and cytosolic proteins were separately extracted and a membrane inserted Na_V *vs* cytosolic Na_V ratio was calculated by using quantification of $Na_V1.4$ and $Na_V1.5$ in membrane and cytosolic fraction. In the control group, membrane $Na_V1.4$ and $Na_V1.5$ were shown to be 90 % and 133 %

respectively more abundant than cytosolic $Na_V1.4$ and $Na_V1.5$ (Figure 5A). Whereas, when IL-13 was perfused for 15 min, those ratios were much greater with an approximately 2.5-fold increase (254 %) for $Na_V1.4$ and 2.5-fold increase (286 %) for $Na_V1.5$ (Figure 5B). All these data suggest that IL-13 may induce the translocation of both $Na_V1.4$ and $Na_V1.5$ from the cytosol to the membrane of the cardiomyocytes, increasing the number of functional channels. In order to determine if $Na_V1.4$ and $Na_V1.5$ translocation was also Ac-dependent, hearts perfused with IL-13 were treated with Ac inhibitor SQ22536. No increase in $Na_V1.4$ and $Na_V1.5$ translocation occurred when perfused hearts were pre-treated with Ac inhibitor; evidencing that the translocation of these two proteins is Ac-dependent (Figure 5C).

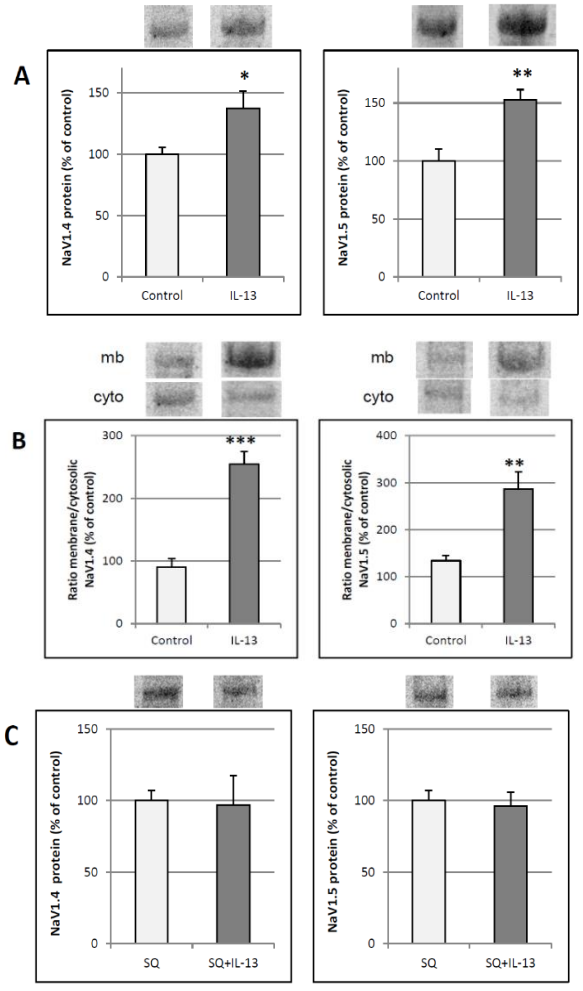


Figure 5: IL-13 effects on $Na_V1.4$ and $Na_V1.5$ expression in cardiomyocyte membrane. (A) after 15 minutes of control conditions, hearts were perfused during 15min with 10ng/ml IL-13 (n=6). Densitometric quantification of Na_V 1.4 and 1.5 membrane expression level was expressed as percentage of control. (B) IL-13 hearts were perfused during 15min with 10ng/ml IL-13 (n=6). Densitometric quantification of membrane *vs* cytosolic protein expression ratio expressed as percentage of control. (C)hearts were perfused during 20min with 100µM of SQ22536 and then with 10ng/ml IL-13 (n=3). Densitometric quantification of membrane to cytosolic protein expression ratio, expressed as percentage compared with control. Data are expressed by mean values ±SEM. Significant differences with *P<0.05 *vs* control, **P<0.01 *vs* control, ***P<0.001 *vs* control.

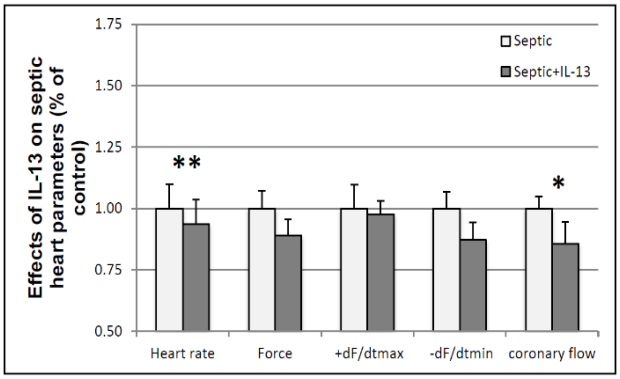
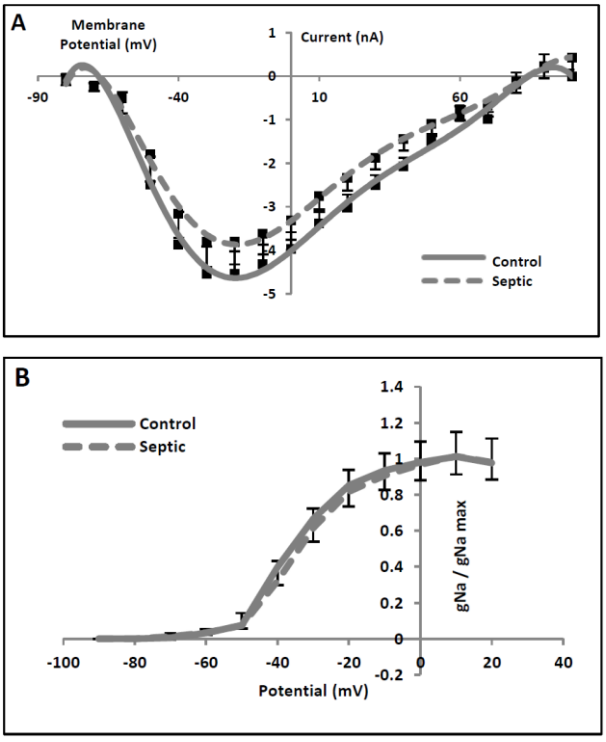


Figure 6: Cardiac function of septic heart before and after IL-13 treatment (n=7). Septic hearts were treated with IL-13 at final concentration 10ng/ml during 15min. Heart rate, Contractile force, maximum speed of contraction (dF/dt max) and relaxation (-dF/dt min), and coronary flow are represented. Each heart represents its own control. Data are expressed by mean values ±SEM. Significant differences with *P<0.05 *vs* control, **P<0.01 *vs* control.*



C	Control	Septic
$I_{Na\ max}$ (nA)	-4.5 ±0.5	-3.8 ±0.5

D	Septic	Septic + IL-13
$I_{Na\ max}$ (nA)	-5.4 ±1.1	-5.7 ±0.9

Figure 7: Sepsis does not induce significant changes in Na currents. (A), sodium current voltage relationship (I/V curves) between control (n=11) and septic (n=11) conditions. (B), activation relationship of sodium current corresponding to the ratio g_{Na}/g_{Namax} (n=11). (C), maximal sodium current in isolated control (n=11) and septic (n=11) cardiomyocytes. (D), maximal sodium current in septic cardiomyocytes before and after IL-13 at 10ng/ml (n=2).

IV IL-13 has no positive nor negative inotropic effect on septic hearts

Our data, so far, suggest that IL-13 has a positive inotropic effect on healthy hearts (Figure 2-5). To determine whether IL-13 has a similar or an opposite effect on septic hearts, hearts from rats undergoing CLP-induced chronic sepsis for 7 days were dissected free and perfused with IL-13 and the experiments were performed, according to the same protocol than used for the healthy hearts. In the opposite of what was observed in healthy hearts, IL-13 showed no effect either on the contractile force or on the contraction and relaxation speeds of the septic hearts (Figure 6). IL-13 even caused a slight but significant decrease of the heart rate (- 6%) and of the coronary flow (- 14%) (Figure 6).

To determine the standard sodium current and activation curves in healthy (control) and septic hearts, macropatch clamp measurements of the isolated ventricular myocytes of septic and healthy hearts were first performed without IL-13. Results showed no significant difference, either in sodium current, with an $I_{Na\ max}$ of -4.5±0.5nA and -3.8±0.5 nA for control (healthy) and septic hearts respectively, or in the activation curve between control (healthy) and septic hearts (Figure 7 A-C). In order to determine the effect of IL-13 on the sodium current of septic heart, macropatch clamp measurements were also performed on septic cardiomyocytes before and after IL-13 infusion following the same protocol than previously stated. Our results showed that IL-13 again has no significant effect on the sodium current with an $I_{Na\ max}$ of -5.4±1.1nA before and -5.7±0.3nA after IL-13 infusion respectively. These results show that, in septic heart, IL-13 positive inotropic effect cannot be evidenced as well as the increase of sodium current. In the opposite a slight negative chronotropic effect is observed.

Discussion

Although the role of IL-13 in many types of illness has been extensively studied, its effect on healthy hearts still remains undetermined. In the present work, we showed that IL-13 induces a positive inotropic effect on healthy rat hearts. The cardiac contractile force and the maximum contraction and relaxation speeds were all increased without affecting the heart rate or the coronary flow. β1-A receptors can be stimulated either by adrenaline/noradrenaline or pharmacological molecules such as isoprenaline. β1-A activation leads to the stimulation of a G stimulatory protein subunit α ($G\alpha_s$), which generates the second messenger cyclic adenosine monophosphate (cAMP) by activation of the Ac. Increasing of cAMP level activates cAMP dependent protein kinase A (PKA), which can phosphorylate a wide range of protein such as phospholamban (PLB) resulting in an activation of SERCA pumps and then an increase in the relaxation speed, troponin I or ryanodine receptor (RyR), strongly involved in cardiac contraction [21]. In addition, PKA also can induce the phosphorylation of dihydropyridine receptor (DHPR) calcium channels thus inducing an increase in calcium current by phosphorylation and hence an increase in contractile force [22]. In fact, IL-13 shows an adrenergic β-1 like effect.

Our results indicate that the same pathway can lead to an increase in sodium currents. Moreover, activation of sodium current by IL-13 may be involved in the inotropic effect. Sodium current is clearly involved in heart contractility. Hearts perfused with Tetrodotoxin, a specific Na^+ channel blocker ($Na_V1.4$-specific), showed a 40 % decrease of contractility compared with their control [23]. In addition, the up-regulation of the sodium current can increase the conduction velocity in non-nodal tissues which is associated with an increase in contraction speed. We demonstrate, by using β1-A receptors - Ac - PKA-specific inhibitors, that IL-13 positive inotropic effect was abolished, suggesting that IL-13 positive inotropic effect occurs via the β1-A - Ac - PKA dependent pathway. When β1-A receptors were inhibited by esmolol, IL-13 induced a decrease of the contractile force and of the contraction and relaxation speeds in the opposite its normal effect. This suggests that another mechanism or pathway may be activated by the cytokine.

Previous studies performed in our laboratory showed that cytokines were able to modify the sodium current in the heart. Pro-inflammatory TNF-α and ciliary neurotrophic factor (CNTF) were shown to decrease the sodium current by activating the PKC pathway [19, 24]. Here, we showed that IL-13 increased $I_{Na\ max}$ by 35%. We demonstrate that IL-13 was responsible for a significant increase in both $Na_V1.4$ and $Na_V1.5$ translocation to the cardiomyocyte membrane. $Na_V1.5$ abundance was greater than the one observed for $Na_V1.4$, thus corresponding to an increase in the $Na_V1.5/Na_V1.4$ ratio. IL-13 induced quickly $Na_V1.4$ and $Na_V1.5$ translocation which supports the hypothesis of a post-translational mechanism. This is in agreement with an up-expression of 37% and 52% for membrane $Na_V1.4$ and $Na_V1.5$ sodium channels respectively. It should also be noticed that the activation curve was shifted toward a more negative potential with $V_{1/2}$ from -46.43 to -50.21 mV by IL-13, which is in agreement with an increase of the $Na_V1.5/Na_V1.4$ ratio in the total channels population by IL-13 in favor of $Na_V1.5$ channels. Previous works in our laboratory demonstrated that the shift toward a hyperpolarized potential in the activation curve corresponded to an increase in to an increase of the ratio $Na_V1.5$ / $Na_V1.4$ as the $Na_V1.5$ channels have an activation potential more negative than $Na_V1.4$ [25]. Moreover, this shift is associated with an increased excitability of the membrane [26].

Previous studies demonstrated a direct link for PKA mediated trafficking of $Na_V1.5$ to the plasma membrane in HEK 293 cell line transfected with $Na_V1.5$ gene [27]. Heart perfused with Ac inhibitor and IL-13 did not show any translocation of either Na_V proteins supporting the hypothesis that the β1-A - Ac - PKA dependent pathway. An increase in the abundance of Na_V proteins, especially of $Na_V1.5$, at the membrane seems to be correlated to a better excitability of the heart. The α subunit of the Na_V can be directly phosphorylated by both PKC and PKA [28]. PKC phosphorylation was shown to cause a decrease in the sodium current [19, 29]. Oppositely, PKA was shown to phosphorylate serine amino acids, such as Ser525 and 528, in the I-II inter-domain linker, leading to a slow increase in the sodium current [30]. Yet, PKA main role in the regulation of sodium current could be the translocation of $Na_V1.5$ to the cell membrane [31]. The positive inotropic of IL-13 can also be due to the PKA activation-induced phosphorylation of the different calcium channels and pumps. However, IL-13 induced no modification of the heart rate. It implies that IL-13 does not induce an increase in cAMP levels in sinus node cells and do not stimulate I_f channels which are responsible for the regulation of the heart rate. Similarly, the type T Ca channels should not be phosphorylated. We can either hypothesize that no IL-13 receptors are present at the surface of sinus node cells or that the PKA activation observed in ventricular cardiomyocytes does not occur in sinus node cells.

In this study, the initial hypothesis was that IL-13 positive inotropic effect could reverse the cardiac dysfunction observed in a chronic sepsis

induced by CLP. However, IL-13 has no positive inotropic effect in septic heart. These findings strongly support previous studies stating that the heart function is impaired during sepsis [2]. Cardiac dysfunction has been linked to the decrease of β1 receptor expression, and the decrease of cAMP production following stimulation by dobutamine [32]. cAMP production has been shown to decrease during myocardial infraction and to be related to the decrease of Ac 5 and 6 isoforms expression [33]. Another explanation would be that the number of IL-13 receptors decreases in the ventricle of septic hearts. However, IL-13 production is increased during sepsis; beside, its inhibition caused a significant decrease of the survival of mice with a seven-day CLP-induced sepsis [12]. This suggests that IL-13 may have a crucial protective role during sepsis. Here, we focused on the rapid effect of IL-13 on the heart contractile force to determine whether IL-13 could be considered as a beneficial cytokine for the heart function. The possible synergic IL-13 action with another mediator of inflammation, such as TGF-β1, and the effects on others tissues and organs studied by Zhou et al. was not investigated in the present study [34]. Similar results were observed with patch clamp on septic cardiomyocytes. IL-13 increased sodium current in the control groups but this effect was lost in septic hearts. Sepsis did not induce any modification in septic sodium current in cardiomyocytes. This can be related to the lack of positive inotropic effect of the IL-13. Both membrane excitability and force contraction are enhanced by PKA activation in healthy hearts. However, PKA activation is impaired during chronic sepsis, which supports the lack of inotropic effect.

Nevertheless, the lack of a positive inotropic effect could be considered as a positive point. Sepsis induces an excessive stimulation of β adrenergic receptors, by increasing plasma catecholamine and then could induce deleterious effects. β1-A stimulation by dobutamine has been shown to increase the mortality rate in critically ill patients [35]. Therefore, the lack of a □1 response to IL-13 could be considered as a protective effect during sepsis. Similarly, selective β1 antagonists have been shown to reduce pro-inflammatory cytokines such as TNF-α, but also to increase heart performance, and improve the survival [36-38].

Conclusion

In this study, we showed for the first time that IL-13 induces a positive inotropic effect on isolated and healthy hearts; but also that the increase in contractile force was associated with an increase in the translocation of voltage-dependent Na^+ channels $Na_V1.4$ and $Na_V1.5$ to the membrane of cardiomyocytes. We also showed that both effects were regulated by the β1-A - Ac - PKA dependent pathway. In addition, IL-13 induces an increase in the heart sodium current which is essential for the excitation/contraction coupling mechanism. Yet, its effect on septic hearts still remains unexplained as IL-13 has no positive inotropic effects on membrane excitability and force contraction. Despite that, IL-13 improves survival during sepsis, but this does not seem to be related to an improvement of the heart function.

Conflicts of interest

The authors declare that no conflict of interest exists. This research was not funded by any private, industrial, public nor non-profit organizations or agencies.

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